EXHIBIT B

2)

1

Repeat with peptide binding Assay.

5 x 10^5 T2 cells / well in RPMI + 5% Boiled FCS.
Add peptides to a final Conc. of 100 μM.
Leave to binding at 37°C 2h
Wash x 1. with PBS + 2% FCS.
Apply mAb HB54. HB117 30' on ice
Wash x 3
Apply Gam FITC (1/250) 30' on ice
Wash x 3
Resuspend cells in PBS (400 μl).

FACS Analysis.

Results. Good.

peptide — 2nd Ab only	Mean: HB54 513	HB117
	37.70	42.32
HPV E7/86-93	109.20	118.12
HBV	146.86	220.67
WT-1 /10-	81.62 ~~93.04~~	144.38 ++
WT-1 /17-	43.79	~~57.04~~ 76.04 +
WT1 /126-	69.78	130.79 ++
WT1 /187-	93.01	136.27 +++
WT1 /225-	70.03	97.56 +
WT1 /235-	95.63	105.90 ++
WT1 /280-	45.66	76.42 +
WT1 /441-	137.52	172.31 +++

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A2 Binding Titration.

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peptides WT1

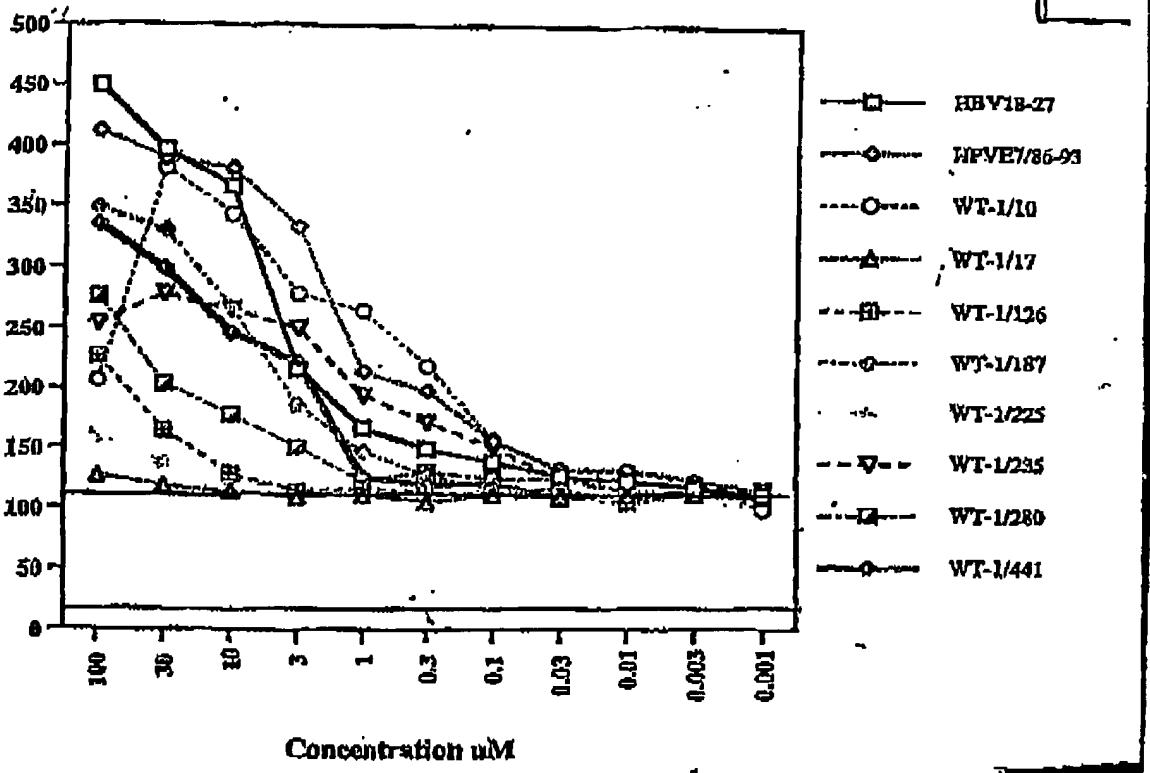
10.
17
126
187
225
235
28
641
HPVE7 E6-93
HBV 17-28

Dilute peptide in RPMI +5% Boiled FCS in 96 well plate (U bottomed). from 100μM of first column to column 11 0.0001 μM) in 50μl medium.
Add 50μl of T2 cells at a conc of 6×10^6/ml ie. 5×10^5/well mix well.
37°C overnight.
wash x1 in PBC (cold) + 1% FCS
stain in HB117 and HB54 (neat culture s/n) 30μl. for 30' on ice.
wash x 3
Apply Ga M Ig FITC at 1/250 (30μl).
for 30' on ice
wash x 3.
Analyse on FACS

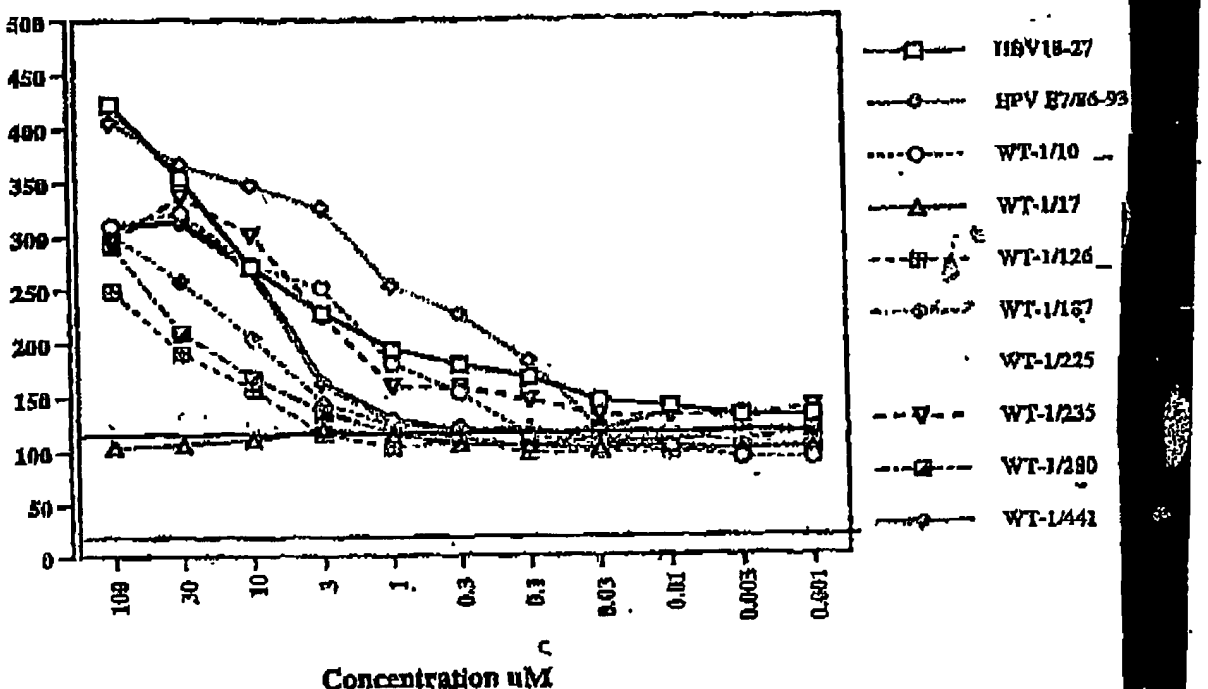
Results: Good see attached sheets.

3

WT-1 peptides binding to A2 molecules on T2 cells
----Stained with HB54 mAb



Binding of WT-1 peptides to A2 molecules on T2 cells
----Stained with HB117 mAb



3.)

4

Setting up Allo- & Self restricted peptide CTL lines

PBMC: LG3 A_2^- Allo

LG4 A_2^+ Self

peptide: WT1 10, 235, 441 and C228 (+ve Control).

Method: as Left — use DC from LG4 as stimulator

Restimulate with peptide loaded CIRA2 cells. (50 μM).

Restimulated 5 " " RMA-S-A2 (mouse).

" "

$ CTL assay

Expand & cloning

Sort of clones

Transfer to a well to 48well plate

Feed

[redacted]

5

Sorting up: Self & Allo-restrict peptide specific CTL line

PBMC LG5 A2+ Self

LG6 A2+ Allo

peptides: WT-1 126. 187. 280
tyr control E7 86-95.

Methods, see previous page.

First round Stimulation with T2 loaded peptides.

Restimulate with peptide loaded-CIR A2

1/2 " " RMA-S-A2

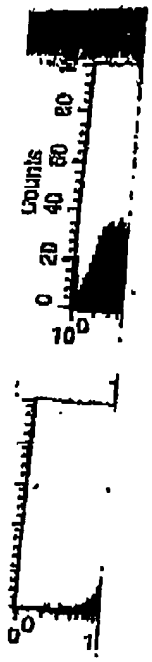
3/4 " " " "

2/3 Restimulation CIR A2

7/3 CTL

2 & 3 Cloning

1 & 4 CTL of clones



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Protocol for peptide specific allorestricted human CTL

1. Prepare stimulator cells: in case of T2 stimulators preincubate o/n 10^6 cells/ml/well in x24 well plates in RPMI medium plus 5% boiled (10 min) FCS with 100 uM peptide. If allo Dc are used as stimulators, allo DC from A2 +ve individuals can be separated as following:

Incubate A2+ PBMC at 37°C O/N, harvest nonadherent cells, resuspend in RPMI+9%FCS at $5X10^6$ /ml and layer onto metrizadime gradient carefully, spin at 1500 for 10 min and harvest the interface (30% low density DC, 20% recovered from PBMC counts). Wash X3 with medium, resuspend at 10 ^{6}/ml, add pepdides to a final conc of 50uM and incubate at 37°C for 4hr. (3000 rad) The stimulator can be added to responders directly w/O washing off peptides.

2. Separate blood by Ficoll centrifugation, wash twice, count, make sure that cells are HLA-A2 negative (by staining with HB54 and HB117 antibodies)

3. Plate $2x10^6$ effectors and $2x10^5$ stimulators per well in x24 well plates in culture medium (RPMI 1640, 10% FCS, 1% glutamine, 1% pen/strept $5x10^{-5}$ M 2-ME) with 500 nM peptide

4. On day 4 prepare stimulators as described in 1. if required. It may be not necessary to preincubate C1RA2 cells for more than 2 hrs

5. On day 5 harvest cells, count spin and seed $5x10^5$ effectors, $2x10^6$ irradiated (about 3000 rad) HLA-A2 negative autologous feeders, $2x10^5$ peptide loaded and irradiated (about 10000 rad) T2 or C1RA2 cells and 500 nM peptide in 2ml of the culture medium with 10u/ml IL-2, 2.5ng/ml IL7 (1/2000 of the stock of 5ug/ml) and with 10% of Q12054 culture sup (anti-CD4 antibodies) (complete medium)

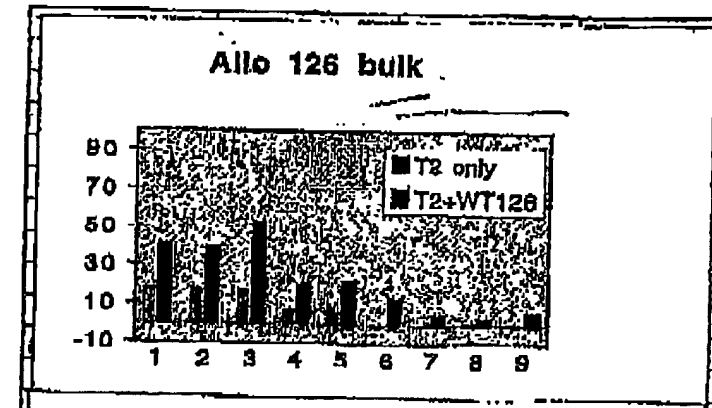
6. 14 days (and further every 2 weeks) later restimulate cultures as described in 5. Cell recovery is usually 200%

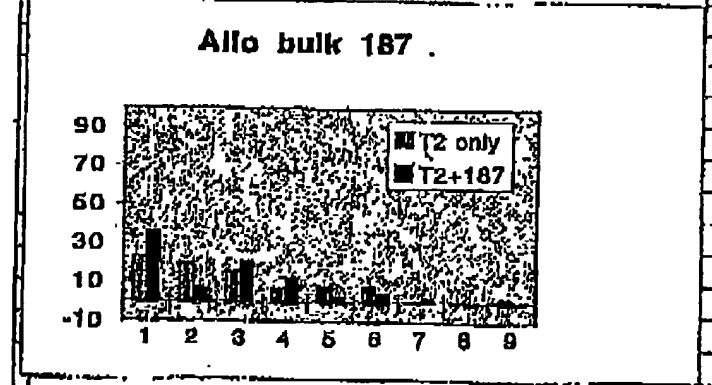
7. On day 5 analyse peptide specificity in a ^{51}Cr release assay using T2 or mouse cells transfected with A2 molecule as CTL targets and cold K562 cells as NK targets (50 per 1 labelled target). Use 0.7% TFA for max release instead of 0.5% SDS. Significant peptide specificity is detectable after 5-7 week in culture with E:T ratio 10:1

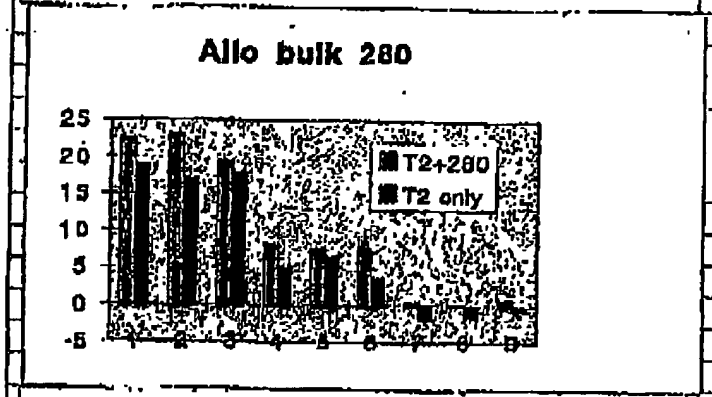
8. Peptide specific cultures can be cloned seeding 1, 10 and 100 cells/well in round bottom x96 well plates in complete culture medium with 10^4

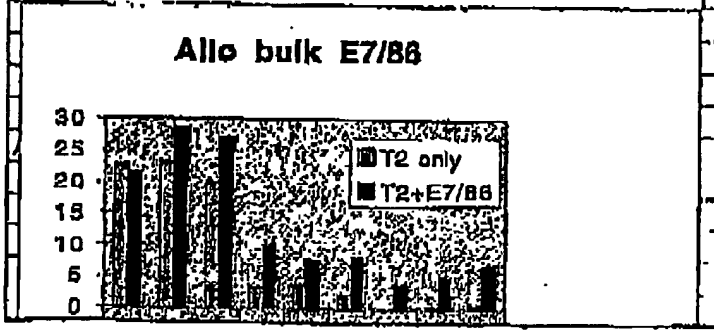
(4)

Sheet1









	T2+128	
224	3199	303
284	3454	288
310	3290	51
300	3343	8
291	3168	
330	3332	
344	3262	
344	3261	
		4
	1534	41
	1508	40
	1870	52
	926	21
	970	22
	708	14
	456	5
	985	3
	492	6

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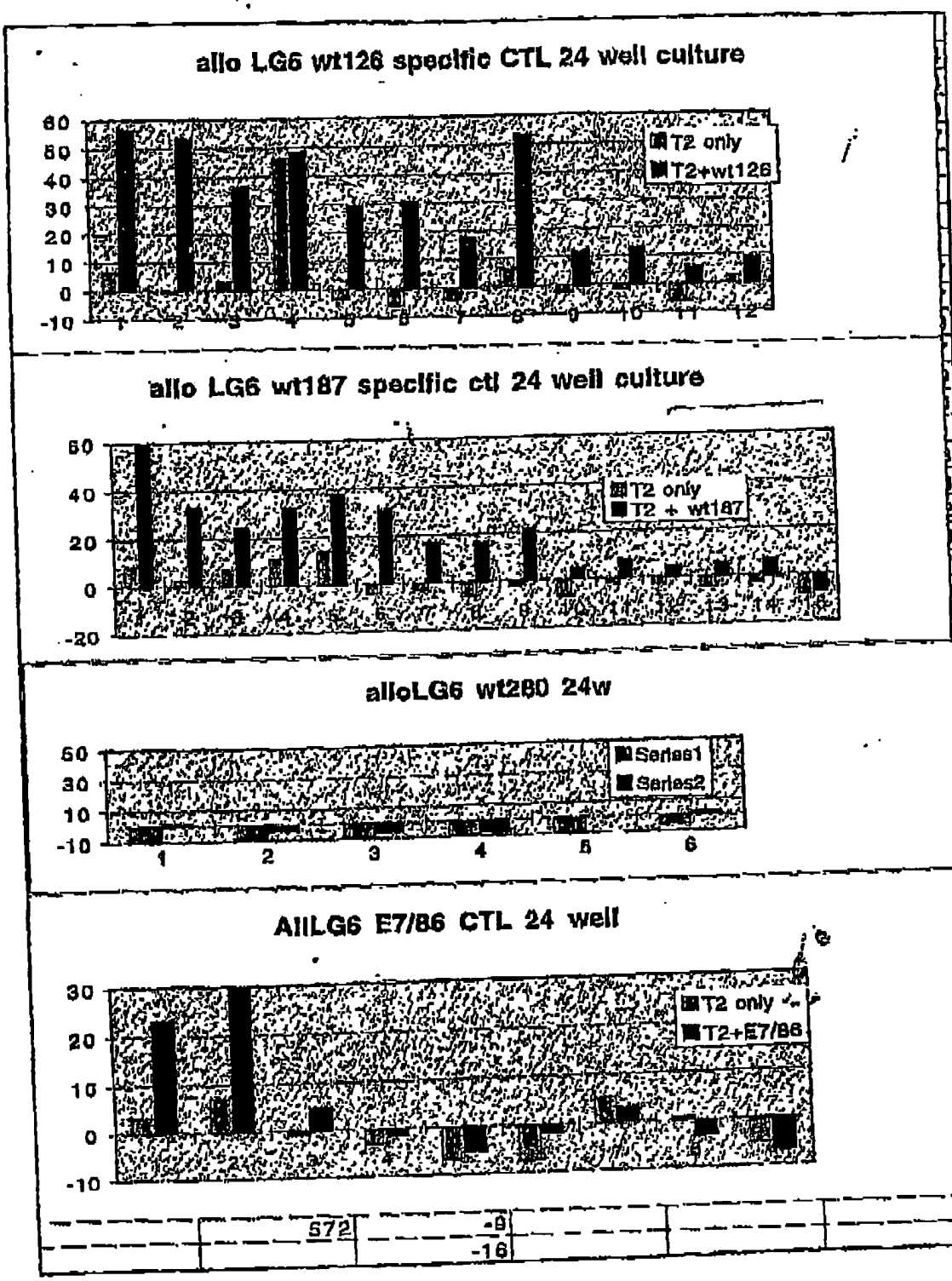
Sheet1


allo LG6 wt126 specific CTL 24 well culture
60
50
40
30
20
10
0
-10
T2 only
T2+wt126
allo LG6 wt187 specific ctl 24 well culture
60
40
20
0
-20
T2 only
T2 + wt187
alloLG6 wt280 24w
50
30
10
-10
1
2
3
4
5
6
Series1
Series2
AllLG6 E7/86 CTL 24 well
30
20
10
0
-10
T2 only
T2+E7/86
572
-8
-16

67

9

51 Cr Release Assay

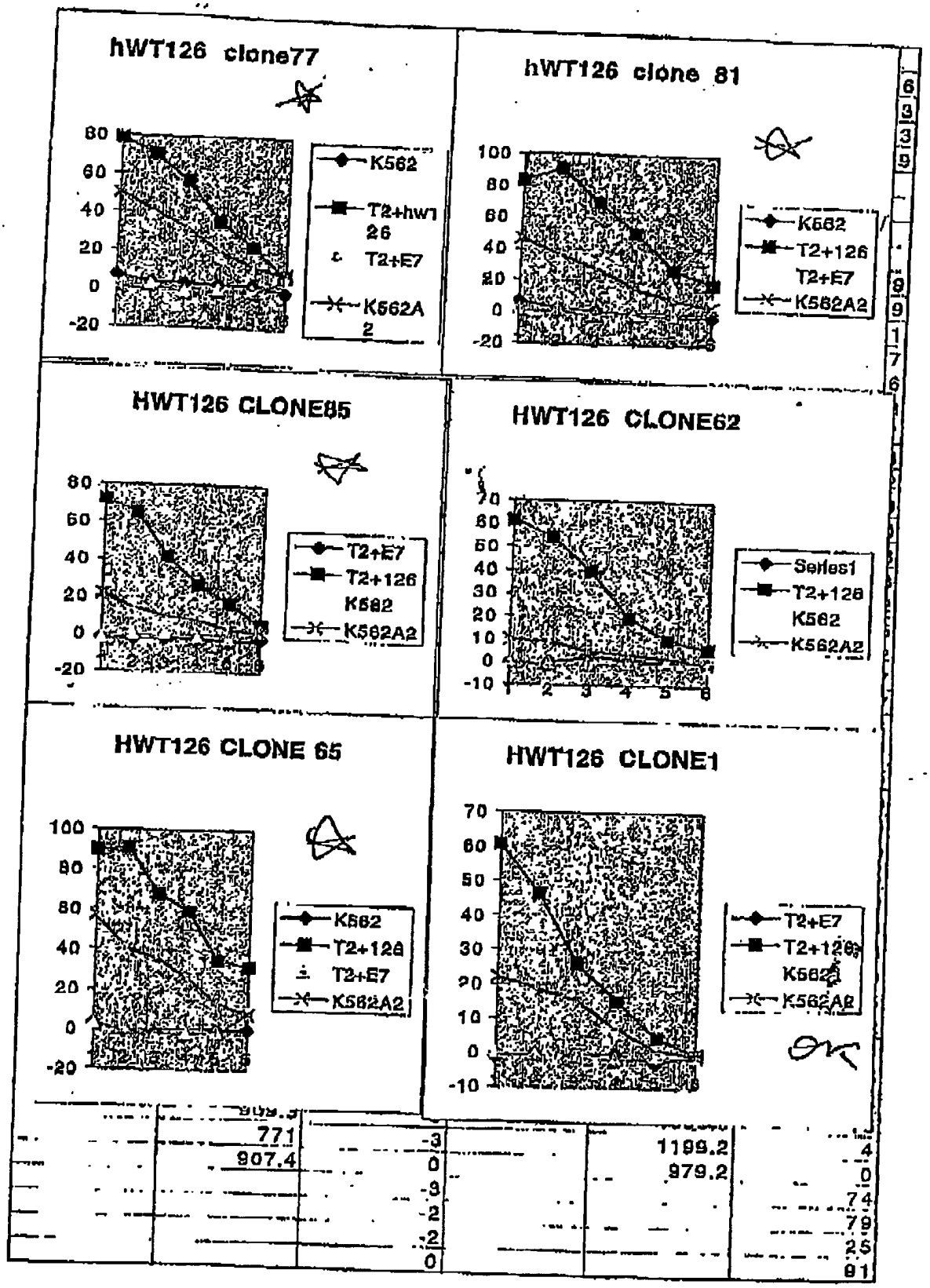
test clones Standard 4 hr

targets T2 + E7
T2 + 126
K + 62
K-62 Az

more tests

10

Sheet1


hWT126 clone77
K562
T2+hwt126
T2+E7
K562A2
hWT126 clone 81
K562
T2+126
T2+E7
K562A2
HWT126 CLONE85
T2+E7
T2+126
K562
K562A2
HWT126 CLONE62
Series1
T2+126
K562
K562A2
HWT126 CLONE 65
K562
T2+126
T2+E7
K562A2
HWT126 CLONE1
T2+E7
T2+126
K562
K562A2

7)

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51 Cr assay

clone 77. 81. 32.

~~target~~ T2+E7 vs. control
T2+126
Bv173.
MV 6041
697
CML Bone Marrow 4 (Foreman)
CD34+ from BM (by Stem)
E:T 25. 12. 6. 3. 1.5 0.75

Results: line 77 line 32. 81

T2+E7	-	+	+
T2+126	+	+	+
Bv173	+	+	+
697	+	+	
MV4041	+	+	+
CML CD34+	+	+	-
CML BMφ	-	±	-

The clone 77 kills CD34+ from CML BM

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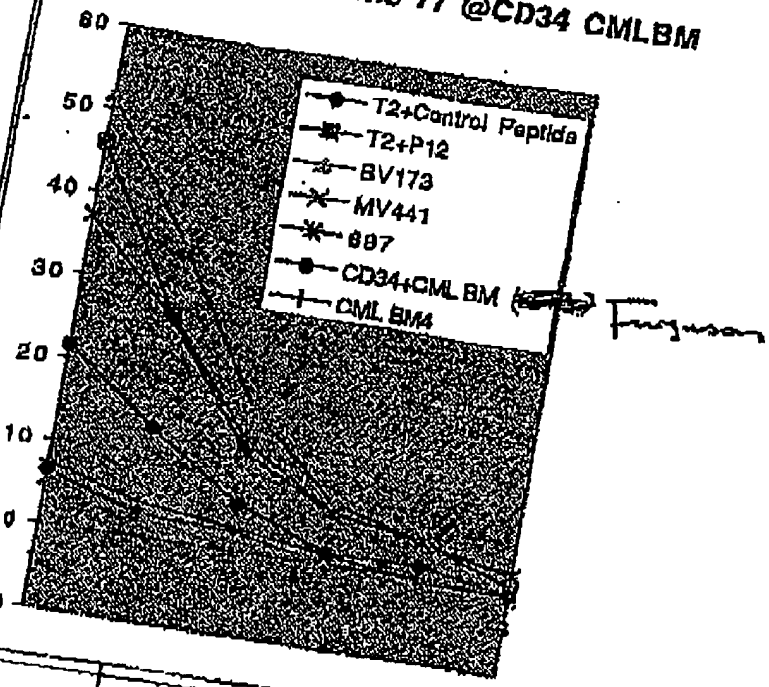

	-1	41
	-4	12
	-1	10
	-1	23
	28	27
	57	76
RNA RT PCR	19	69
	50	28
+	-2	80
++	-2	0
	-2	11
+	-4	60
	-2	68
+	-1	40
	67	31
	13	97
	6	97
	3	100
	0	105
	-5	19
	-6	117
	-7	99
	13	100
	-3	109
	-5	25
		85
		102
		61
		86
		99
		87
		12
		61
		25
		101
		88
		124
		81
		85
		26
		116
		26
		28
		46
		86
		69
		1
		76

697
BV173
Ferguson cD34+
Thomas
Godfrey